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Inhibition of Complement-Mediated Cytolysis by the Terminal Complement Inhibitor of Herpesvirus Saimiri

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Herpesvirus saimiri (HVS) is a lymphotropic herpesvirus that induces T-cell transformation in vitro and causes lymphomas and leukemias in New World primates other than its natural host, the squirrel monkey. Nucleotide sequence analysis of the HVS genome revealed two open reading frames with significant homology to genes for human complement regulatory molecules. One of these genes encodes a predicted protein (designated HVSCD59) with 48% amino acid sequence identity to the human terminal complement regulatory protein CD59 (HuCD59). The CD59 homolog from squirrel monkey (SMCD59) was cloned, and the corresponding amino acid sequence showed 69% identity with HVSCD59. BALB/3T3 cells stably expressing HVSCD59, SMCD59, or HuCD59 were equally protected from complement-mediated lysis by human serum. However, only HVSCD59-expressing cells were effectively protected from complement-mediated lysis when challenged with rat serum, suggesting that HVSCD59 was less species restrictive. The complement regulatory activity of HVSCD59 and SMCD59 occurred after C3b deposition, indicating terminal complement inhibition. Treatment of BALB/3T3 stable transfectants with phosphatidylinositol-specific phospholipase C prior to complement attack decreased the complement regulatory function of HVSCD59, suggesting cell surface attachment via a glycosyl-phosphatidylinositol anchor. Cells expressing HVSCD59 effectively inhibited complement-mediated lysis by squirrel monkey serum in comparison with SMCD59-expressing cells. Finally HVSCD59-specific transcripts were detected in owl monkey cells permissive for lytic HVS replication but not in T cells transformed by HVS, which failed to produce virions. These data are the first to demonstrate a functional, virally encoded terminal complement inhibitor and suggest that HVSCD59 represents a humoral immune evasion mechanism supporting the lytic life cycle of HVS.

Various mechanisms of host immune evasion have been described for several families of viruses (16). One such mechanism is through the perturbation of the complement cascade. Examples of potential antiviral activities mediated by the complement system include lysis of free virus or virally infected cells, viral opsonization or neutralization, and the elicitation of specific inflammatory immune responses. Consequently, some viruses have developed specific strategies to avoid complement-mediated destruction. For example, the major secreted protein of vaccinia virus (vaccinia virus complement control protein) binds C3b and C4b, inhibiting both the classical and alternative complement pathways (19–21, 26). Additionally, glycoprotein C1 (gC1) of herpes simplex virus type 1 functions as a receptor for C3b on virally infected cells and inhibits complement-mediated lysis (13, 14, 18).

Herpesvirus saimiri (HVS), a member of the gammaherpesvirus subfamily, causes lymphomas and leukemias in New World primates excluding squirrel monkey, its natural host (12). Sequence analysis of the HVS genome (group A, strain 11) has revealed two additional candidates for viral complement regulatory genes (2). One of these putative regulatory molecules, termed the complement control protein homolog (CCPH), shows significant homology with a family of human complement regulatory proteins known to interact with C3b or C4b (1). The CCPH gene is differentially spliced, resulting in both a membrane-bound and a secreted form of the protein from HVS-infected cells (1). Expression of both a membrane-bound and a soluble protein through differential splicing is also characteristic of decay-accelerating factor (DAF), a complement regulatory protein that interacts with C3b (8, 15, 27).

A second open reading frame in the HVS genome (HVS-A15) codes for a predicted protein with significant homology (48% identity) to the human complement regulatory protein CD59 (HuCD59) (3). CD59 is an 18-kDa glycoprotein that is attached to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor (34, 37). CD59 regulates complement-mediated membrane damage by preventing activation of C9, thus restricting assembly of the cytolytic pore (34). CD59-mediated complement regulation exhibits species restriction and is most effective when the C8 and C9 components of the membrane attack complex are derived from primate sera (35). CD59 also participates in T-cell adhesion and activation through its interactions with CD2, a T-cell-specific glycoprotein (17).

In this study, we investigated the complement regulatory potential of the HVS-A15 gene product (HVSCD59). Concomitantly, the cloning and expression of SMCD59, the CD59 homolog from squirrel monkey (the natural host of HVS), allowed a detailed structural and functional comparison between SMCD59 and HVSCD59. We show that HVSCD59 and SMCD59 expressed in BALB/3T3 cells rendered these cells resistant to complement-mediated membrane damage and that complement regulation most likely occurs at the level of the membrane attack complex. These results represent the first demonstration of a virally encoded terminal complement inhibitor and suggest a mechanism of immune evasion for HVS.

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MATERIALS AND METHODS

HVSCD59, SMCD59, and HuCD59 clones. The prototype strain 11 of HVS subgroup A (28) was originally isolated from peripheral blood of a squirrel monkey (11). Nucleotide sequencing of the HVSCD59 gene (HVS-A15) was initially done as described elsewhere (3). SMCD59 was cloned by PCR amplification of first-strand cDNA, using oligonucleotides constructed from regions flanking the start and stop codons of the HuCD59 sequence (5′-GAAGGGATCCCGGGCGCC GCCAGGTCTGTGGACAATCAC-3′ and 5′-GGGAGAA GCTCTCCTGGTGTTGAC-3′, respectively). Total RNA was isolated from squirrel monkey lung cells (ATCC CCL 194) by the acid guanidinium technique (10). Five micrograms of total RNA was heated at 65°C for 3 min and cooled on ice prior to first-strand cDNA synthesis for 1 h at 37°C in a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.25 mM each deoxynucleoside triphosphate (dNTP), 0.5 μg of oligo$(dT)_{16}$, and 20 U of avian myeloblastosis virus reverse transcriptase (Seikagaku of America, Inc., Rockville, Md.). Five microliters of the cDNA pool was used as a template in a 100-μl PCR (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 0.1% [wt/vol] gelatin, 1.0% Triton X-100, 200 μM each dNTP, 2.5 U of *Taq* DNA polymerase [Perkin-Elmer-Cetus, Norwalk, Conn.]), using 25 pM each primer. Thermal cycler conditions were as follows: 30 cycles of 95°C for 1 min, 42°C for 1 min, and 72°C for 1 min followed by 72°C for 10 min. The PCR fragment was cloned into the pCRII vector of the T/A cloning kit as instructed by the manufacturer (Invitrogen, San Diego, Calif.). DNA sequence analysis of double-stranded DNA was performed by the dideoxy-chain termination method (36). SMCD59 cDNA sequence was verified by sequencing two additional isolates generated from separate PCRs. The 9-bp nucleotide insertion found in SMCD59 cDNA was confirmed by sequence analysis of SMCD59 exon 3. One microgram of genomic DNA isolated from squirrel monkey lung cells (24) was PCR amplified by using primers derived from the 5′ and 3′ ends of the putative exon 3 in squirrel monkey (predicted from the location of exon 3 in HuCD59 [33]). PCR amplification, cloning, and sequence analysis were performed as described above. HuCD59 in pUC19 was provided by A. Bothwell (Yale University). HVSCD59, SMCD59, and HuCD59 cDNAs were subcloned into a pcDNA expression vector (pcDNA I/Amp or pcDNA3; Invitrogen) for eukaryotic cell expression.

BALB/3T3 stable transfections. The HVSCD59, SMCD59, and HuCD59 constructs (20 μg) were linearized with *Pvu*I and then subjected to phenol-chloroform extraction and ethanol precipitation. In the case of HVSCD59, the pcDNA I/Amp construct was coprecipitated with the selection vector pSV2Neo. BALB/3T3 cells (ATCC CCL 163) at 50% confluency were transfected by the calcium phosphate method as previously described (4). Stable transfectants were selected with DME/High Modified (JRH Biosciences, Kansas City, Mo.) containing 1 mM sodium pyruvate, 2 mM L-glutamine, and 500 μg of G418 per ml.

PCR analysis. Transcription of HVSCD59, SMCD59, and HuCD59 cDNAs in BALB/3T3 clones was assessed by PCR amplification of reverse-transcribed total RNA. RNA isolation and reverse transcription were done as described above. PCR amplification of first-strand cDNAs was performed by using 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min. Oligonucleotides used in the amplification of SMCD59 and HuCD59 clone cDNAs were identical to those described for the original cloning of SMCD59. The oligonucleotide pair used for amplification of the HVSCD59 clone cDNA was derived from regions inside the HVSCD59 coding region (5′-GCGC GGATCCTTGCAATGCTACAACTGTTC-3′ and 5′-GCG CGAATTCTTACTAATTTTCAATCCCTTTGTTCAC-3′).

Complement-mediated killing assay. Complement-mediated killing of BALB/3T3 cells transfected with HVSCD59, SMCD59, HuCD59, or vector alone was assessed by release of the cytoplasmic indicator dye calcein AM (Molecular Probes, Inc., Eugene, Ore.). Transfected cells were grown to confluency in 96-well plates, washed twice with Hanks balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA) and incubated with 10 μM calcein AM at 37°C for 30 min. Cells were again washed twice before the addition of polyclonal anti-BALB/3T3 immunoglobulin G (IgG; 2 mg/ml) for a 30-min incubation at 23°C. The anti-BALB/3T3 IgG was generated by injecting rabbits with intact BALB/3T3 cells suspended in complete Freund's adjuvant. After unbound IgG was washed away, complement components were deposited by adding whole human, squirrel monkey, or rat serum at various concentrations for a 30-min incubation at 37°C. Dye release was measured on a Millipore Cytofluor 2350 plate reader (excitation, 490 nm; emission, 530 nm). Percent dye release was calculated relative to total cell-associated dye determined from cells treated with 1% sodium dodecyl sulfate. Dye release from cells not subjected to serum was used to determine background fluorescence and nonspecific dye release. In the experiment to determine the sensitivity of HVSCD59 to phosphatidylinositol-specific phospholipase C (PIPLC), cells were treated with PIPLC (Boehringer Mannheim, Indianapolis, Ind.) at 1 U/ml prior to the addition of calcein AM and serum.

C3 deposition analysis. BALB/3T3 cell transfectants were suspended in HBSS with 1% BSA and incubated with anti-BALB/3T3 IgG (2 mg/ml) for 30 min at 4°C. After two washes in HBSS, 20% human C8-deficient (C8d) serum (Quidel, San Diego, Calif.) was added for a 30-min incubation at 37°C. Cells were washed once before addition of anti-C3d monoclonal antibody (Quidel) at 5 μg/ml for a 30-min incubation on ice. Following a final wash, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (15 μg/ml) for 30 min on ice and analyzed on a Becton Dickinson FACSort. Nonspecific binding of anti-C3 antibody was determined in the absence of serum.

Northern (RNA) blot analysis. Polyadenylated mRNA from mock-infected and HVS-infected owl monkey kidney (OMK) cells or growth-transformed marmoset or human T cells was purified from 75 μg of total RNA by using oligo(dT)-Dynabeads (Dynal, Hamburg, Germany). The following T-cell lines were used: (i) 1670, derived from marmoset spleen transformed with HVS A11, nonproducer (25); (ii) P1079, peripheral blood lymphocytes (PBL) from *Callitrix jacchus* transformed with HVS C-488, virus producer (31a); (iii) P1084, human T cells isolated from thymus transformed with HVS C-488, $CD8^+$, nonproducer (6); (iv) 3C, HVS C-488-transformed human PBL, $CD8^+$, nonproducer (11a); and (v) CB15, HVS C-488-transformed human cord blood lymphocytes, $CD4^+$, nonproducer (6). The Northern blot was hybridized with a PCR fragment generated from HVS strain C-488, using the following primers: 5′-GGTAATGTATATTTTGTTTA CGTTGGT-3′ and 5′-CTTTTAAAGAGGAAAGTTCCA AGCAGT-3′. The blot was hybridized in 50% formamide at 30°C overnight and subsequently washed four times at 50°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min, four times with 1× SSC for 15 min, and once with 0.2× SSC for 15 min. The same membrane was stripped and rehybridized with a probe derived from a housekeeping gene (the glyceraldehyde 3-phosphate dehydrogenase gene) to determine relative RNA amounts between lanes.

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                 ------------signal peptide-------------+1
SMCD59     MGI QGG SVL FGL LLV LAV FCH SGN SLQ CYS CPL PTM ESM
HVSCD59          M YI* *T* V*T F-* **K PIH *** **N *SH S** ---
HuCD59     *** *** *** *** *** *** *** **H *** **N **N **A ---

SMCD59     ECT AST NCT SNL DSC LIA KAG SGV YYR CWK FDD CSF KRI
HVSCD59    Q** T** S** *** *** *** *** *** *** *** *** *** ***
HuCD59     D*K TAV **S *DF *A* **T *** LQ* *NK *** *EH *N* NDV

                                             ∇ ------putative GPI
SMCD59     SNQ LSE TQL KYH CCK KNL CNV KEV LEN GGT TLS KKT ILL
HVSCD59    *** *** *** *** *** *** *** NKG I** IKR *I* D*A -**
HuCD59     TTR *R* NE* T*Y *** *D* **F N*Q *** *** S** E** V**

           anchor signal--------
SMCD59     LVT PFL AAA WSR HP
HVSCD59    *LA L** VT* *NF PL
HuCD59     *** *** *** **L **
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FIG. 1. Sequence comparison of HVSCD59 and primate CD59 homologs. The deduced amino acid sequences for HVSCD59, SMCD59, and HuCD59 are shown in the one-letter amino acid codes. Amino acid identities relative to SMCD59 are indicated by asterisks. The first amino acid of the mature protein (identified for HuCD59) is shown as +1, and the putative GPI anchor attachment site is indicated by a triangle. N-linked glycosylation motifs are shown as shaded boxes. Gaps inserted to align the sequences are marked by dashed lines.

RESULTS

Sequence comparison of viral and mammalian CD59 homologs. An open reading frame identified in HVS encodes a putative protein sharing 48% amino acid sequence identity with HuCD59 (3). To determine the divergence of the viral protein, the CD59 homolog was cloned from squirrel monkey, the natural host of HVS. Using oligonucleotides derived from regions flanking the start and stop codons of HuCD59, SMCD59 was PCR amplified from first-strand cDNA. The primers were chosen on the basis of the high degree of homology found between HuCD59 and HVSCD59 in these regions. The SMCD59 cDNA nucleotide sequence consisted of a 393-bp open reading frame beginning with a putative start codon and terminating with a stop codon (data not shown; GenBank accession number L22859). An alignment of the predicted amino acid sequences of SMCD59, HVSCD59, and HuCD59 is shown in Fig. 1. The comparison revealed an overall sequence identity of 69% between SMCD59 and both HVSCD59 and HuCD59. The SMCD59 leader peptide and GPI anchor signal showed 42 and 40% identity, respectively, to HVSCD59, while the putative mature protein was 86% conserved. In contrast, the signal peptide and hydrophobic tail were highly conserved between SMCD59 and HuCD59, with identities of 96 and 85%, respectively, while the mature protein sequence was only 55% conserved. The N-linked glycosylation site, shown to be critical for function in HuCD59 (32), was conserved in SMCD59. Although this glycosylation site is absent in HVSCD59, an alternate N-linked glycosylation motif occurred at amino acid position 5 of the mature protein. The most likely site for CD59 GPI anchor attachment based on GPI anchor signal commonalities (23, 29–31) was conserved in HVSCD59, SMCD59, and HuCD59.

An additional nine base pairs were identified in SMCD59 cDNA, which encodes three amino acids in the mature peptide not found in HVSCD59 or HuCD59. To rule out cloning artifacts, the presence of the additional nucleotides was confirmed through PCR amplification and sequencing of SMCD59 exon 3 from genomic DNA (data not shown).

Expression of CD59 homologs in BALB/3T3 cells. Expression plasmids containing HVSCD59, SMCD59, and HuCD59 were transfected into BALB/3T3 cells for functional analysis. HuCD59-expressing clones were identified by FACS analysis using an anti-HuCD59 polyclonal serum or anti-HuCD59

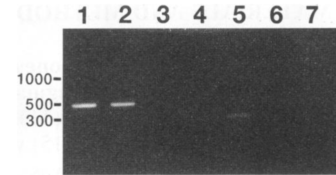

FIG. 2. PCR analysis of BALB/3T3 stable transfectants. Total RNA isolated from cells transfected with HVSCD59, SMCD59, HuCD59, and vector alone was reverse transcribed and PCR amplified with insert-specific oligonucleotides. Lanes: 1, 2, and 5, amplification of a single product from HuCD59, SMCD59, and HVSCD59 clones, respectively; 3 and 6, amplification of first-strand cDNA from the vector-alone control cells; 4 and 7, amplification reactions containing all reagents except first-strand cDNA. Numbers at the left indicate DNA size in base pairs.

monoclonal antibody, but anti-HuCD59 antibodies did not cross-react with HVSCD59 or SMCD59 (data not shown). To identify clones expressing these molecules, PCR analysis was performed. Total RNAs were isolated from BALB/3T3 clones, and first-strand cDNAs were generated. The oligonucleotides used to PCR clone SMCD59 were used to PCR amplify SMCD59 and HuCD59 cDNAs, while two internal oligonucleotides derived from HVSCD59 were used to amplify HVSCD59 cDNA. A representative positive clone for each construct is shown in Fig. 2. A product of about 450 bp was amplified from the SMCD59 and HuCD59 clones, while amplification of the HVSCD59 clone yielded a product of approximately 275 bp, the sizes expected from PCR utilizing these primers (Fig. 2, lanes 1, 2, and 5, respectively). Amplification of cDNA from a clone transfected with vector alone and reactions performed in the absence of first-strand cDNAs yielded no products (lanes 3, 4, 6, and 7). Positive clones expressing HVSCD59, SMCD59, and HuCD59 at the transcriptional level were chosen for functional analysis.

Complement regulatory activity of HVSCD59 and SMCD59. Stably transfected BALB/3T3 clones were tested in a dye release assay for protection against lysis by human complement. Cells were loaded with the cytoplasmic dye calcein AM, and membrane pore formation was measured as a function of dye release following exposure to complement. Cells expressing HVSCD59, SMCD59, or HuCD59 were equally effective in inhibiting membrane damage by human complement (Fig. 3A). For example, cells expressing these molecules were fourfold more effective in preventing complement-mediated lysis at 10% human serum than cells transfected with the Neo vector alone. Cells transfected with the Neo vector alone were readily lysed.

HuCD59 preferentially restricts lysis of cells by human complement with decreased or no inhibitory activity against complement from various other species, including rodent (35, 38). To address whether HVSCD59 and SMCD59 also exhibit species restriction, BALB/3T3 cells expressing these molecules were tested for resistance to rat complement. BALB/3T3 cells expressing HVSCD59 were protected from complement-mediated cytolysis by whole rat serum, while cells expressing SMCD59 and HuCD59 were complement sensitive (Fig. 3B). Using 10% rat serum as a representative point, HVSCD59-expressing cells were fourfold more effective in inhibiting membrane damage than HuCD59-expressing cells and fivefold more effective than cells expressing SMCD59 or cells transfected with the Neo vector alone. Additional clones positive for expression of HVSCD59 or SMCD59 by PCR analysis were

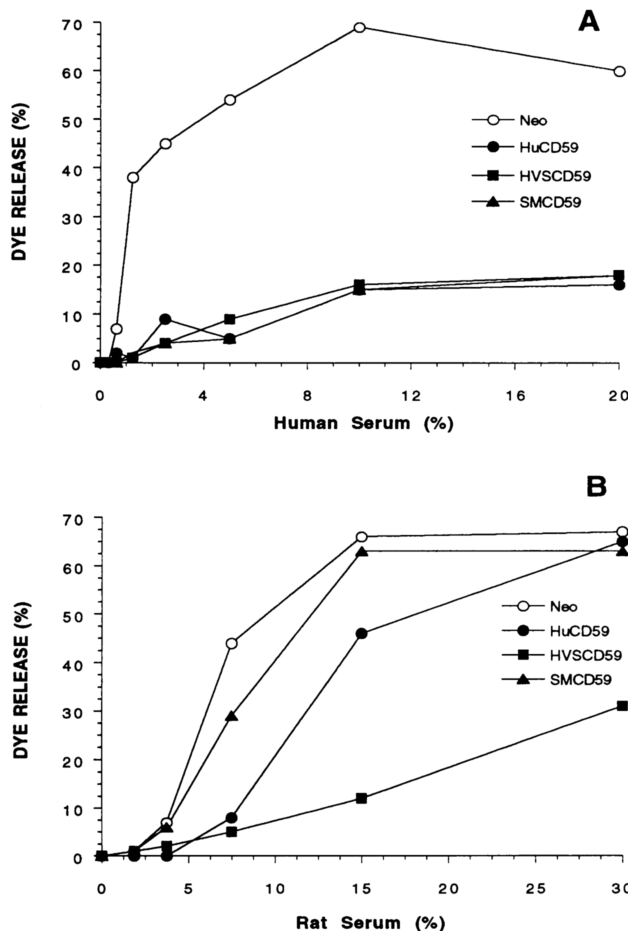

FIG. 3. Complement regulatory activities of HVSCD59, SMCD59, and HuCD59. Stable transfectants expressing HVSCD59, SMCD59, or HuCD59 at the transcriptional level were subjected to a complement lysis assay. Complement-mediated damage was measured by the percent dye released following the addition of complement relative to the total amount of dye taken up by the cells. In each panel, the percent dye release is shown on the ordinate and the percent serum used is represented on the abscissa. The stable BALB/3T3 transfectants shown are the Neo vector control, HuCD59, HVSCD59, and SMCD59. The source of complement used in each case was human serum (A) or rat serum (B). Data shown are means of duplicate determinations from a single experiment, one of four so performed.

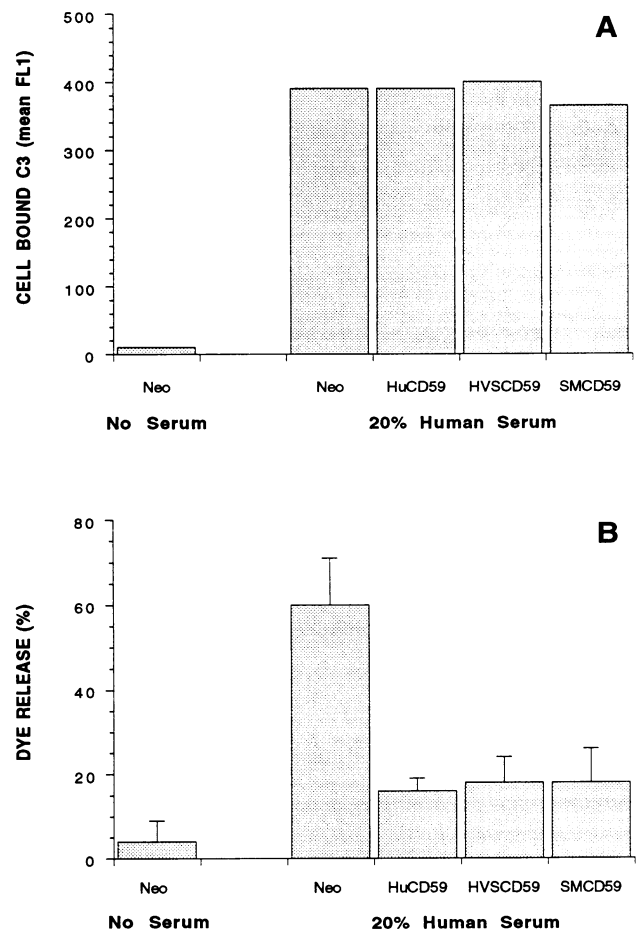

FIG. 4. C3 deposition analysis. HVSCD59-, SMCD59-, and Hu CD59-expressing cells were incubated with C8d human serum prior to incubation with anti-C3 antibody and FACS analysis (A). Bars denote mean fluorescence channel of 10,000 events analyzed for C3 binding after exposure to 20% C8d human serum. Nonspecific binding of the anti-C3 antibody was determined on the Neo vector-alone transfectant in the absence of C8d human serum. The same transfectants were subjected to the dye release assay (see Materials and Methods), using 20% human serum as a source of complement (B). Bars represent mean percentages of dye release relative to total cellular dye. Nonspecific dye release was determined on the Neo vector-alone transfectant in the absence of C8d human serum. Each error bar represents the standard deviation of triplicate determinations from a single experiment, one of four so performed.

shown to have similar complement regulatory activities (data not shown).

C3 deposition analysis. The high degree of homology between the HVSCD59 and HuCD59 genes strongly suggests a role for HVSCD59 as a terminal complement inhibitor. To rule out complement regulation before the initiation of the terminal complement cascade, transfected cells expressing HVSCD59 and SMCD59 were tested for C3 deposition relative to cells transfected with HuCD59 or Neo vector alone. The same cells were subjected to complement-mediated lysis by human complement to examine the degree of complement-mediated cytolysis relative to the level of C3 deposition. C3 deposition on the surface of all transfectants, including the Neo vector alone, was equal (Fig. 4A). However, cells expressing HVSCD59, SMCD59, and HuCD59 released less than 20% of the cytoplasmic dye when subjected to 20% human serum, while cells transfected with the Neo vector alone released 60% (Fig. 4B). This finding indicated that the complement regulatory activity of HVSCD59 and SMCD59 occurs after C3 deposition.

PIPLC sensitivity of HVSCD59. Comparison of HVSCD59 with SMCD59 and HuCD59 revealed considerable variability in the signal peptide and hydrophobic domains. These regions are known to be critical for GPI anchoring (7, 9), the mechanism of membrane attachment demonstrated for HuCD59 (34). To investigate membrane attachment characteristics of HVSCD59, transfectants were incubated with PIPLC prior to exposure to complement. The complement regulatory activities of cells expressing HVSCD59 or HuCD59 were substantially reduced (55 to 75%) by PIPLC treatment (Fig. 5A). PIPLC treatment of control cells had no significant effect on the complement sensitivity of these cells. Cells expressing

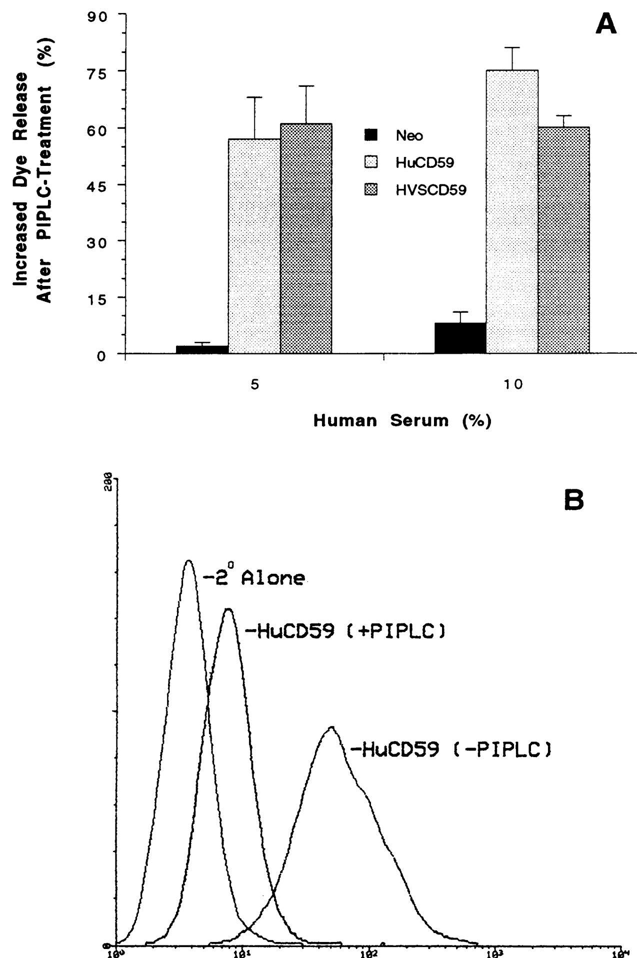

FIG. 5. PIPLC sensitivity of HVSCD59. HVSCD59 and HuCD59 stable transfectants were treated with PIPLC prior to the addition of human complement in the complement-mediated killing assay. The effect of PIPLC treatment was measured as the percent increase in dye release following PIPLC treatment (ordinate) at 5 or 10% human serum (abscissa). (A) Effects of PIPLC treatment on the complement regulatory activity of cells expressing the Neo vector control, HuCD59, and HVSCD59. Each bar represents the mean of triplicate determinations in a single experiment, one of three so performed. Error bars denote standard errors. (B) FACS profile of HuCD59-expressing cells stained with anti-CD59 antibody before and after PIPLC treatment.

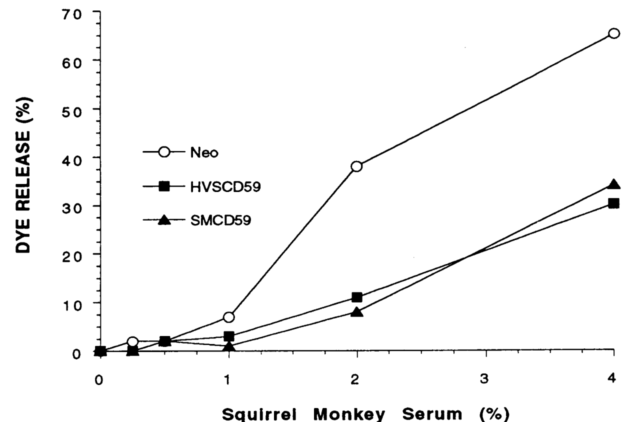

FIG. 6. HVSCD59 complement inhibition against squirrel monkey serum. HVSCD59- and SMCD59-expressing cells were subjected to a complement-mediated killing assay as described for Fig. 3. The stable BALB/3T3 transfectants shown are the Neo vector control, HVSCD59, and SMCD59. The source of complement used was squirrel monkey serum. Each point of the curve is an average of two separate determinations. Data represent a single experiment, one of two so performed.

HuCD59 were FACS analyzed with anti-CD59 antibody before and after PIPLC treatment to assess the efficiency of CD59 removal from the BALB/3T3 cell surface. The majority of HuCD59 was removed from the surface of cells by PIPLC, as the mean fluorescence of cells following PIPLC treatment approached that seen with cells incubated with secondary antibody alone (Fig. 5B). Since available antibodies to HuCD59 do not cross-react with HVSCD59, FACS analysis for surface expression of this molecule was not possible. These data suggested that the reduction in complement regulatory activity by HVSCD59-transfected cells following PIPLC treatment was due to removal of the protein from the cell surface.

HVSCD59 complement inhibition against squirrel monkey serum. Squirrel monkey is the natural host of HVS, and the virus can be readily isolated from its peripheral mononuclear blood cells (11). To investigate a potential complement regulatory role for HVSCD59 in the squirrel monkey, HVSCD59-expressing cells were tested for complement inhibition against squirrel monkey serum. HVSCD59 effectively inhibited membrane damage mediated by squirrel monkey complement (Fig. 6). Cells expressing these molecules were fourfold more effective in preventing complement-mediated lysis by squirrel monkey serum than cells transfected with the Neo vector alone. Cells expressing SMCD59 showed the same level of complement protection demonstrated with HVSCD59-expressing cells. These data establish efficient complement regulatory activity by HVSCD59 against serum from its natural host, suggesting a mechanism of immune evasion for HVS.

Transcription of HVSCD59 in HVS-infected cells. BALB/3T3 cells stably transfected with HVSCD59 were protected from complement-mediated lysis. This observation suggests a mechanism for immune evasion during the life cycle of the virus. To investigate this hypothesis further, transcription of the HVSCD59 gene was examined in lytically infected cells. Poly(A)$^+$ mRNA isolated from cells lytically infected or transformed with HVS was analyzed by Northern blotting with an HVSCD59 PCR fragment (C-488) probe. Specific transcripts of 5.0, 3.7, 2.8, and 2.0 kb were detected in RNA isolated from OMK cells lytically infected with HVS subtype A, B, or C (Fig. 7, lanes 2, 3, and 4, respectively). Corresponding transcripts were detected in RNA isolated from the semipermissive marmoset PBL cell line p1079 (lane 6). HVS-transformed cell lines that do not produce virus, including marmoset spleen 1670, human PBL P1084, human PBL 3C, and human cord blood lymphocyte CB15, were negative for HVSCD59-specific transcripts (lanes 5, 7, 8, and 9, respectively). Uninfected OMK cells hybridized with a nonspecific 1.5-kb band common to all RNA populations examined (lane 1). The multiple transcripts hybridizing with the C-488 probe probably reflect run-through transcription products from the HVSCD59 gene and/or adjacent genes. However, hybridization of the C-488 probe under these stringent conditions indicates HVSCD59-specific sequence. These results demonstrate that the HVSCD59 gene is actively transcribed in lytically infected cells and provide evidence for potential surface expression of HVSCD59 on virally infected cells and/or the viral envelope.

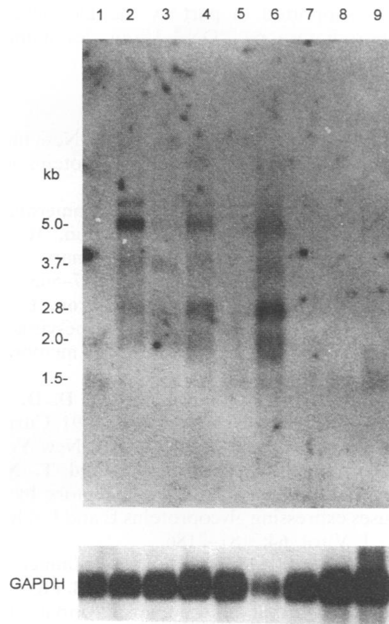

FIG. 7. Northern blot analysis of HVS-infected cells. Poly(A)$^+$ RNA was isolated from HVS-infected or growth-transformed cells for Northern blot analysis using a PCR fragment from HVS C-488 specific for the gene 15 open reading frame as the hybridizing probe. Lane 1 contains mRNA from mock-infected OMK cells; lanes 2 to 4 contain mRNA from OMK cells infected with HVS strains A11, B-SMHI, and C-488, respectively. Other lanes contain mRNA from marmoset cell lines 1670 (lane 5) and P1079 (lane 6) and human cell lines P1084 (lane 7), 3C (lane 8), and CB15 (lane 9). The mRNA size of each hybridizing band is shown at the left. The lower panel shows the pattern of hybridization using a probe to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, showing relative amounts of mRNA loaded in each lane.

DISCUSSION

In this report, we describe the functional characterization of a terminal complement regulatory protein homolog (HVS CD59) encoded by a gene identified in HVS. Cloning and sequence analysis of the CD59 homolog in squirrel monkey, the natural host of HVS, allowed sequence and functional comparisons between HVSCD59, SMCD59, and HuCD59. These proteins were shown to inhibit complement-mediated membrane damage when expressed on the surface of BALB/3T3 cells. The complement regulatory activity of HVSCD59 and SMCD59 occurred after C3 deposition, indicating that the molecules function as terminal complement inhibitors. Complement inhibition by HVSCD59 appeared to lack species restriction, as only HVSCD59-expressing cells were significantly protected from whole rat serum. PIPLC treatment of BALB/3T3 cells expressing HVSCD59 reduced the ability of these cells to protect against human complement attack, suggesting that the molecule is attached to the membrane via a GPI anchor. Finally, cells lytically infected with HVS were shown to transcribe the HVSCD59 gene.

BALB/3T3 cells expressing HuCD59 and SMCD59 were effectively protected against human complement but not rat complement. This result parallels previous studies showing that HuCD59 restricts lysis of cells by human complement but is ineffective at inhibiting cell lysis when a rodent source of complement is used (35, 38). Complement protection of cells expressing HVSCD59 was not restricted to primate sera, as these cells were also resistant to complement-mediated damage by rat serum. This observation was unexpected given the high degree of sequence conservation between the HVSCD59 and SMCD59 mature proteins (86% identity). One possible explanation for the lack of species restriction demonstrated with HVSCD59 is the change in location of a putative N-linked glycosylation site. It has been reported that the asparagine residue at amino acid position 18 is the only glycosylation site in the HuCD59 mature protein and that deglycosylation of the molecule drastically reduces its complement regulatory activity (32). This unique N-linked glycosylation site is also found in SMCD59 as well as other primate CD59 homologs, including baboon, African green monkey, owl monkey, and marmoset proteins (data not shown; GenBank accession numbers L22863, L22862, L22861, and L22860, respectively). Although HVSCD59 lacks this putative glycosylation site, it appears that an alternative N-linked glycosylation site may exist at amino acid position 5. Whether the alternative glycosylation site is glycosylated and necessary for function is unknown. Recent analysis of the HVS-15 nucleotide sequence from HVS subgroup B (HVS-B15) indicates that the predicted protein from this gene does not contain a putative N-linked glycosylation site (11b). If the HVS-B15 gene product is capable of regulating complement, N-linked glycosylation of the viral CD59 homolog may not be necessary for complement regulatory activity. Whether changes in the glycosylation pattern of HVSCD59 correlate with the lack of species restriction characteristic of this molecule remains to be determined.

Protein sequence comparison between HVSCD59 and SMCD59 revealed three amino acid residues in the squirrel monkey mature protein not found in the HVSCD59 amino acid sequence. This finding was confirmed by sequence analysis of genomic SMCD59 exon 3. This result was unexpected considering the high degree of conservation that exists between the two molecules throughout the mature protein. Furthermore, sequence analysis of other New World primate CD59 homologs, including those of owl monkey and marmoset, indicates that the additional amino acids are unique to squirrel monkey (data not shown). These results suggest that the HVSCD59 gene may have been captured from the squirrel monkey prior to the acquisition of the additional nucleotides or that HVSCD59 was not captured from the squirrel monkey but rather was derived from another member of the New World primates earlier in the evolution of the virus. In any case, SMCD59 retained its ability to regulate complement.

The high degree of sequence conservation between the HVSCD59 and SMCD59 mature proteins was not observed between the signal peptide or hydrophobic tail. Conversely, recent sequence comparisons between CD59 homologs identified in New World and Old World primates show high conservation of the signal peptide and hydrophobic tail, while the mature peptide is more divergent (for an example, see Fig. 1). The effects of these changes on the processing and/or function of HVSCD59 are not known. Although the amino acid residues in the hydrophobic tail were divergent in HVSCD59, most of these changes were conservative and the overall features of a putative GPI anchor signal were maintained (23, 30, 31). A study involving the GPI anchor signal of DAF showed that scrambling the amino acid residues in the hydrophobic domain did not affect the ability of the molecule to be GPI anchored (9). Our studies demonstrate that PIPLC treatment of cells expressing HVSCD59 prior to complement challenge reduced the ability of these cells to inhibit complement-mediated membrane damage. This finding suggests that HVSCD59 is attached to the cell surface via a GPI anchor moiety. Although GPI-anchored proteins have not been de-

scribed for virally encoded proteins, a recent report has shown that the addition of the DAF-GPI anchoring signal to bovine herpesvirus type 1 gIII enabled the expression of the molecule on the surface of the virus (22). Taken together, these results suggest that HVSCD59 may function as a complement regulatory protein on the surface of the cell and/or viral particle.

Interactions between human CD59 and the T-cell-specific glycoprotein CD2 have recently been described (17). Considering this interaction and the predilection of HVS for T cells in vivo, it is intriguing to consider CD2 as a potential HVS receptor, a molecule not yet identified. Although HVSCD59 has not been shown to be present on the envelope of the virion, the presence of a putative signal peptide and hydrophobic tail and the conservation of complement regulatory activity suggest a protein targeted for surface expression. Interactions between HVSCD59 and CD2 are currently being investigated.

An additional open reading frame found in the HVS genome codes for a protein that shares homology and structural organization with a family of complement regulatory proteins that act at the level of C3b and C4b (1). This protein, designated CCPH, is expressed as a secreted molecule in HVS-infected cells and as membrane-bound virion surface component. CCPH has recently been shown to inhibit complement-mediated membrane damage of stably transfected BALB/3T3 cells at the level of C3b (12a). We show that HVSCD59 regulates complement after C3b deposition. Considering this finding and the conserved structural features between HuCD59 and HVSCD59 (3), it is likely that HVSCD59 functions as a terminal complement inhibitor. Although HVSCD59 has not definitely been shown to be expressed on the envelope of HVS, it seems likely that CCPH and HVSCD59 act in a dual complement regulatory mechanism at different points in the complement cascade. Multiple host immune evasion mechanisms are not unprecedented. The herpes simplex virus not only encodes gC, which inhibits both the alternative and classical complement pathways (18), but also expresses gE-gI, which binds the Fc portion of immunoglobulin G, inhibiting complement lysis and possibly Fc receptor-mediated phagocytosis (5). The presence of both HVS CD59 and CCPH on the virus envelope would provide resistance to complement-mediated virolysis in the bloodstream as well as prevent opsonization via specific complement receptors on phagocytes.

The gammaherpesvirus group is known to persist in PBL (12). Both HVSCD59 (this report) and CCPH (1) have been shown to be transcribed during HVS infection, and CCPH appears to be expressed on the surface of virally infected cells. Although squirrel monkey cells are already protected from autologous complement attack by endogenous regulatory molecules (i.e., SMCD59), expression of heterologous complement regulatory proteins may enhance protection from lysis of virally infected cells. Increased protection of cells due to the level of expression of a complement regulatory molecule was recently shown in CHO cells stably transfected with HuCD59. Transfected cells expressing HuCD59 showed increasing levels of protection against human serum complement as the level of HuCD59 increased (38). Complement regulation of virally infected cells through increased expression of complement regulatory proteins may protect cells marked for immune destruction.

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